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How Might We Get from Genes to Circuits to Disease?

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Abstract

Recent advances in the identification of risk genes for psychiatric disorders have set the stage for functional interrogation of disease related-circuits and underlying mechanisms of pathophysiology. Still, investigators face significant challenges: (a) hundreds of genes may contribute to pathogenesis of a given disorder (polygenicity and genetic heterogeneity), (b) risk alleles may only cause the disease in combination with other factors (reduced penetrance), and (c) commonly used rodent models may have significant limitations in studying psychiatric disorders, due to differences in brain structure and function between rodents and humans.

To address these challenges, high-throughput functional assays should be developed in combination with induced pluripotent stem cells (iPSC) technology and novel genome-engineering technologies, as these will help identify common pathways and mechanisms onto which multiple risk genes may converge. To overcome limitations of current animal models in psychiatric research, novel genome-editing technologies provide an opportunity to generate better animal models (e.g., the common marmoset) to dissect disease-relevant circuit dysfunction. Finally, powerful new tools (e.g., CLARITY, dense neural circuit reconstruction, and optogenetics) may help identify and test the relationship between distinct circuit defects and abnormal behaviors observed in these animal models. Such approaches are needed to span the gap between emerging genetic information and the symptomatic description of neuropsychiatric disorders.

Introduction

Large-scale genetic studies of psychiatric disorders are identifying an increasing number of risk genes for psychiatric disorders, such as autism and schizophrenia (Craddock and Sklar 2013; Giusti-Rodríguez and Sullivan 2013; Kendler 2013; McCarroll and Hyman 2013; McCarroll et al. 2014; Owen 2012a,b; Rosti et al. 2014). For the first time in history, it is now possible to study, with high confidence, a large number of genes associated with psychiatric disorders. Although genetics is not the only factor that contributes to the

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development of psychiatric disorders, it is one of the most important factors, given the high heritability of these diseases (Cardno et al. 1999). Thus, the recent discovery of high-confidence risk alleles that are being mapped to disease-associated genes provides an unprecedented opportunity for neuroscientists to dissect neurobiological mechanisms of psychiatric disorders.

The genetic data, however, also present several challenges. The first concerns polygenicity and genetic heterogeneity. Current data suggest that hundreds of genes may cause or contribute to the pathogenesis of each common psychiatric disorder. An important step is therefore to identify potential converging pathways and mechanisms that are shared by the multiple risk alleles. The second challenge is the limited penetrance of each risk allele. Each risk allele has a small effect and acts in combination with other genetic and nongenetic risk factors to produce disease symptoms. Thus, identifying the multiple genetic variants and nongenetic risk factors that render a particular risk allele pathogenic will be critical to the successful modeling and subsequent understanding of the disorder. Another challenge is that genetic data do not directly provide cell type-specific information. Systematic mapping of cell type-specific effects of risk alleles on gene expression and function will be essential for dissecting their pathogenic roles. Finally, although protein-coding DNA sequences are fairly conserved through evolution, regulatory sequences are far less conserved. Moreover, it is difficult to model deficits of higher brain function in animals such as rodents, due to the inherent differences in brain structure, function, and physiology between species. The recent development of new genome-editing technologies enables genetic manipulation in many species (Boch et al. 2009; Cong et al. 2013; Mali et al. 2013b; Zhang et al. 2011) and thus may lead to the generation of better animal models for psychiatric research. In addition, other new technologies for mapping and probing circuit connectivity and function will greatly facilitate the study of neural circuit mechanisms of psychiatric disorders.

From Risk Genes to Converging Pathways and Mechanisms

Although psychiatric disorders are genetically heterogeneous, each disorder has certain defining behavioral abnormalities, such as social communication deficits in autism and psychosis and cognitive impairment in schizophrenia. This suggests that heterogeneous genetic factors converge on certain common mechanisms controlling these behaviors. Bioinformatic analyses of genetic data are beginning to reveal clustering of risk genes to certain signaling pathways or cellular domains (e.g., postsynaptic density). This convergence of action could happen at multiple levels, including molecular and cellular pathways, circuit connectivity, and network dynamics.

Mapping a large number of risk genes onto functional pathways requires medium- to high-throughput assays to measure neuronal functions at multiple

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levels, including protein-protein interaction, neuronal morphology, synaptic function, and neuronal connectivity. While gene knockdown approaches have traditionally employed RNA interference or morpholinos in cultured neurons and model organisms (e.g., *Caenorhabditis elegans*, *Drosophila*, and zebrafish) to investigate gene function, new genome-editing technologies (e.g., TALEN and CRISPR) provide highly efficient ways to make precise genetic manipulations in a variety of systems (Fontes and Lakshmipathy 2013; Mali et al. 2013a; Hsu et al. 2014). By introducing the exact variants from human patients, these new technologies allow the interrogation of functional consequences and directionality (loss or gain of function) for each risk allele. Importantly, genome editing by these new technologies is not limited to coding sequences; this allows risk alleles to be investigated with sequence variants in noncoding regions, which may affect epigenetic modification, transcription, and splicing.

Multiple assays, such as high-density imaging (Sharma et al. 2013), multielectrode arrays (McConnell et al. 2012), and calcium imaging (Chen et al. 2013), can be used to detect changes in signaling pathways, neuron morphology, synapse number, receptor trafficking, synaptic transmission, and neuronal activity. However, we do not have scalable assays that would allow us to detect circuit-specific defects, which might be a key converging mechanism. One possible solution is to use embryonic stem cell-derived organoid culture (Eiraku et al. 2008; Lancaster et al. 2013) to develop local circuits for functional analysis. For some evolutionary conserved circuits, it is also possible to use simpler model organisms (e.g., zebrafish) for high-throughput disease-relevant circuit assays (Stewart et al. 2014).

The lack of brain tissues from patients has been a major obstacle in psychiatric research. With the rapid advance in iPSC technology (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Zhang et al. 2013), it might soon be possible to develop functional assays using human embryonic stem cells or iPSC-derived neurons (Imaizumi and Okano 2014). When combined with the CRISPR genome-engineering technology, these assays will likely permit functional studies of diverse risk alleles, both individually and in combination. Although promising, further development is needed to resolve several key issues, including the lack of robust methodologies to produce distinct subtypes of neurons and glia efficiently. Another main issue to be addressed is the immature nature of induced pluripotent stem cells, which is evident through the paucity of spines and synapses in the derived neurons.

How to Dissect Polygenic Mechanisms

Recent genome-wide association studies have identified a large number of risk alleles. Each risk allele likely has a small effect and acts in combination with other genetic and/or nongenetic factors. Thus, identifying other genetic

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and nongenetic factors that render a particular risk allele pathogenic is key to understanding the disorder.

One possible approach is to build protein interactomes involving the risk genes. This can be achieved by combining proteomic analysis of protein complexes and bioinformatic analysis of existing protein-interaction databases. Once the interactome is established, risk alleles (individually or in combination) could then be tested for their effects on the assembly, and possibly the function of the protein complexes. Such data would provide a framework to identify other genes that function in the same pathway as the risk gene, and thus are more likely to work in concert with the risk gene. Ultimately, these proteomic studies may reveal novel molecular and cellular mechanisms of gene function and yield potential drug targets.

The human brain contains a very large number of neuronal and glial subtypes. Each subtype displays unique structure, connectivity, and function that arise from, or are mediated by, distinct patterns of gene expression, signaling pathways, electric properties, and neurotransmitter utilization. Having new genomics and related technologies available that offer precise profiling of gene expression and cellular function means that hosts of diverse cell types are likely to be identified in the coming years. Combining this approach with interactome studies will be an exciting but challenging step toward the understanding of cell-specific interactomes.

Another promising approach is to use genome-wide screens to identify functional modifiers of risk genes. This will require the development and validation of medium- to high-throughput assays for risk allele-associated cellular phenotypes. One could then use whole-genome RNAi knockdown or CRISPR knockout approaches to perform the genome-wide screen to identify modifier genes of cellular phenotypes of risk alleles. The outstanding strength of this approach is that it not only aims at the interactions of one gene product with another, but also may identify parallel pathways that converge on regulating the same cellular function, and thus help to build a "functional interactome," which may be the molecular correlate of polygenic diseases.

Genome-wide mutagenesis screens for genetic modifiers of gene functions have been successfully used in model organisms such as *C. elegans*, *Drosophila*, and zebrafish (St. Johnston 2002; Kettleborough et al. 2013). For risk genes that are evolutionarily conserved, this approach can be very powerful. In the light of high costs, however, mutagenesis screens of phenotypic enhancers in mice, even for a handful of risk genes, are not realistic. A new approach is to take advantage of the large resource of new mutation- or variant-carrying inbred mouse strains created at the Collaborative Cross (Churchill et al. 2004). By systematically crossing mutant mice harboring risk alleles with various inbred mouse strains bidirectionally (i.e., either to ameliorate or exacerbate), genetic modifiers of particular phenotypes can be rapidly mapped and identified.

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Together, combined large-scale data from these approaches may be able to define the biological pathways that contribute to the polygenic nature of psychiatric disorders. These proteomic and functional gene-gene interaction data would also be extremely helpful in interpreting rare variant data from exome and whole genome sequencing. Once the genetics and pathways of polygenic traits are identified, it will be possible to truly model these disorders in cells and animals, and then to take advantage of these valid models in drug screening.

Better Animal Models for Circuit Analysis

Due to the limited access to patient brain tissue, animal models will continue to play key roles in understanding how disease-associated molecular and cellular changes lead to neural circuit dysfunction underlying clinically relevant cognitive and behavioral abnormality. Currently, genetically engineered mice are the most commonly used model organism; they have been very informative for studying neural circuits and brain regions that are structurally and functionally conserved in evolution, such as amygdala function in fear memory (LeDoux 2014) and the basal ganglia circuit in repetitive behavior (Welch et al. 2007; Graybiel 2008). However, modeling deficits of higher brain function, such as mood and cognition, has been difficult in rodents due to significant differences in the structure and physiology of the brains between humans and rodents. One such example is the significantly smaller size of the prefrontal cortex in rodents compared to that of humans. Crucially, structural and functional defects in dorsolateral prefrontal cortex of the brain of schizophrenia patients are considered an important cause of working memory deficits (Volk and Lewis 2010). This anatomical structure is unique to primates (Preuss 1995), making rodent studies difficult to interpret. Indeed, the lack of suitable preclinical models for brain disorders has been considered a major obstacle to the development of new drugs for CNS diseases such as autism, depression, Alzheimer disease, and many others. Thus, there is an urgent need to develop better animal models that more closely capture human pathophysiology.

The development of highly efficient new genome-editing technologies, such as TALEN and CRISPR, has enabled genetic manipulation in primates (Liu et al. 2014; Niu et al. 2014), raising the possibility of generating primate models for psychiatric disorders. Decades of nonhuman primate research have been central to our understanding of physiological brain function. Most such work is done with rhesus macaques, but because of their size, cost, and long generation time, it is not ideal to use macaques as a routine genetic model. An attractive alternative is the common marmoset *Callithrix jacchus* (Carrion and Patterson 2012). Marmosets are New World monkeys; they are highly social with a strong family structure, and show complex vocal behaviors that are seen as an expression of and model for social communication and social cognition (Schiel and Huber 2006; Dell'Mour et al. 2009; Miller et al. 2010; Takahashi

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et al. 2013). From a practical and genetic engineering perspective, the common marmoset has considerable advantages: small body size and low weight (~350 g), comparatively early sexual maturity at 12–18 months, and biannual births which produce 2–3 offspring from each pregnancy.

The neuroanatomy of the common marmoset is well described. Like macaques, but unlike rodents, marmosets have a well-developed prefrontal cortex, a region that is critical for many cognitive functions that are impaired in human psychiatric disorders. The small size and smooth surface of the marmoset brain are also experimentally advantageous for neuroscience research. Although less studied than macaques, the marmoset has been a popular model organism for studies of auditory physiology. With its fast reproductive cycle, the common marmoset has the potential to anchor the next generation of genetically engineered model organisms for brain disorder research (Okano et al. 2012).

New Technologies for Circuit Analysis

In addition to advances in the genetic analysis of psychiatric disorders, ongoing development of new technologies for probing circuit connectivity and functionality will also greatly facilitate the dissection of neurobiological substrates of psychiatric disorders. These technologies include long-range circuit imaging techniques, cellular resolution connectomics, and optogenetic tools for precise spatiotemporal manipulation of neural activity.

CLARITY

Functional interrogations of complex biological systems are particularly challenging since primary defects, which occur on the molecular and cellular level, may produce symptoms and impairments at the level of neural circuits. Understanding the full nature of the pathophysiology thus requires the integration of high-resolution molecular and cellular observations with functional imaging of neural circuits in their native three-dimensional structure.

A recently developed technique, termed CLARITY, meets this need by enabling the preservation of the three-dimensional framework of the mouse brain while rendering the entire tissue optically transparent (Chung et al. 2013). The method removes the light-scattering lipid bilayers from the brain and replaces them with a new physical framework composed of a tissue-hydrogel hybrid, which facilitates the penetration of both light and macromolecules such as antibodies and *in situ* hybridization probes (Chung et al. 2013). Using CLARITY, biochemical information can be obtained in the context of cellular structure and long-range connectivity with single cell resolution. These unique qualities of CLARITY could provide new invaluable insights into differences in specific circuit structure and wiring between normal and diseased brains. For example, preliminary results from CLARITY revealed abnormal ladder-like dendritic

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arborizations in parvalbumin-positive interneurons in an autism postmortem brain (Chung et al. 2013). In addition, new insights may originate from functional integration of *in vivo* activity recording with calcium imaging or from optogenetic manipulation of specific neuronal populations with *post hoc* analysis of their respective long-range connectivity.

Dense Neural Circuit Reconstruction Using Electron Microscopy

To gain insight on a level of neuronal ultrastructure in a network context, high-resolution two-dimensional imaging using either serial block face scanning electron microscopy (SBF-SEM; Briggman et al. 2011) or automated serial-section tape-collection scanning electron microscopy (ATUM-SEM; Hayworth et al. 2006) followed by three-dimensional reconstruction can be performed. These labor-intensive approaches allow reliable detection of cell bodies, neurites, and synapses. They thus yield cellular resolution connectomics data, which may eventually shed light on how dysfunction of distinct neural circuits arises from genetic variants linked to psychiatric disease.

Specifically, genetically engineered rodent models for these disease conditions could be subject to sequential two-dimensional imaging and three-dimensional reconstruction to reveal altered connectivity on microcircuits. These analyses could potentially lead to the identification of new distinct cell types that have been previously missed by lower-resolution approaches, such as sparse labeling and fluorescence microscopy. Identification of these subclasses may be of great importance to unravel subtle differences in neural architecture in disease conditions. In addition, while classical labeling methods cannot provide the three-dimensional information needed to track synapses back to one neuron involved in the connectivity with another, dense neural circuit reconstruction allows a count of the number of synapses on a specific neuron as an indicator of the strength of interaction. Furthermore, since electron microscopy permits the number of vesicles at a given synapse to be determined, not only the number of synapses but also the strength of interaction can be evaluated. Given that altered information processing in psychiatric disorders may arise from dysfunctional wiring and skewed synaptic interaction strength, high-resolution connectomics information, when generated with high throughput, would greatly improve our understanding of these conditions.

Optogenetics

After risk-associated genes are identified and valid animal models generated, brain areas that underlie the pathophysiology can be identified using correlative approaches, such as brain activity marker labeling, calcium imaging, and electrophysiology. Just as the observation of the genetic association has to be causally supported by the generation of an animal model, so too must the observation that an underlying circuit appears to be abnormally active, and

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therefore potentially driving abnormal behavior, be tested through experimental manipulation. Optogenetics, a technology based on light-activated ion channels, enables researchers to conduct these functional interrogations by controlling defined circuits in a bidirectional temporally and spatially precise manner (Boyden et al. 2005). Importantly, transgenic mouse lines with cell type-specific opsin expression or specific Cre-driver mouse lines that can be transduced with conditional opsin-encoding adeno-associated viruses allow precise manipulation of select neural subsets (Zhao et al. 2011). In addition to cell type-specific control, high spatial precision allows not only the stimulation of distinct anatomical regions but also projection-defined activity control. Thus, observational resolution is improved, since a given nucleus in the brain may send long-range projections to multiple downstream regions with different, potentially even oppositional, downstream effects. For example, while stimulation of region-defined cell populations in the basolateral amygdala elicits anxiogenic effects in accordance with previous findings, high-resolution manipulation using projection-specific activation of lateral basolateral amygdala to central amygdala connections revealed an anxiolytic effect of this circuit (Tye et al. 2011). In an experimental setting, one can employ retrograde rabies- or herpes-virus based approaches to transduce nerve terminals at a projection site and then focally stimulate the respective upstream region. Alternatively, transduction methods based on adeno-associated viruses can be used in a defined region to study projection-specific effects after opsin transport into axon terminals and focal activation of the terminals in a given downstream region (Tye and Deisseroth 2012).

Another exciting optogenetic approach takes advantage of bistable kinetics, a novel class of engineered opsin displays. These bistable channels conduct cations upon illumination with blue light and remain open for extended periods of time, on the order of tens of minutes, until conductance is terminated with a flash of yellow light (Yizhar et al. 2011). The key advantage is that activating bistable opsins does not readily trigger spiking, but rather causes subthreshold depolarization. Given that specific circuits in psychiatric diseases may neither be fully shut off, nor in a highly hyperactive state, and that we lack knowledge regarding spiking representations of behavioral state features, bistable opsins are particularly valuable in studying the facilitation of endogenous activity in defined circuits in a minimally artificial manner. In addition, more complex behavioral tasks can be performed upon detachment of the optical fiber or even minimally invasive transcranial opsin activation, which is possible in rodents due to enhanced light sensitivity of bistable opsins (Yizhar et al. 2011).

Further interesting optogenetic tools available to study disease-related circuits are the recently developed opsins Chronos und Chrimson, which allow independent stimulation of two distinct neural populations at a time (Klapoetke et al. 2014). While both Chrimson and Chronos extend the rich toolbox of activating optogenetic tools, there is a strong demand for more refined silencing tools than halorhodopsin and ArchT. Intriguingly, comprehensive remodeling

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of a cation-conducting channelrhodopsin variant resulted in chloride-conducting opsins for neural silencing with high temporal resolution (Wietek et al. 2014; Berndt et al. 2014). Building on these findings, further molecular engineering extending the optogenetic toolbox with opsins that display desired properties, such as high light sensitivity or bistable kinetics, can be anticipated. Applying these new tools to circuit studies in models for neuropsychiatric diseases, ideally nonhuman primate models which are being developed (Liu et al. 2014), will substantially inform our understanding of these debilitating conditions.

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